Acaricide resistance and synergism between permethrin and amitraz against susceptible and resistant strains of *Boophilus microplus* (Acari: Ixodidae)†

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Abstract: The control of the southern cattle tick, *Boophilus microplus* (Canestrini), in Mexico and many other countries relies on chemical acaricides. *Boophilus microplus* has developed resistance to all major classes of acaricides in recent years. To gain a better understanding of the resistance and to develop resistance management strategies that benefit both Mexican ranchers and USDA's cattle fever tick eradication program (CFTEP), the authors used larval bioassay techniques to determine levels of resistance to permethrin and amitraz and then evaluated synergism between these two acaricides in one susceptible laboratory tick strain and four resistant strains originating from Mexico and Brazil. To examine mechanisms of resistance to permethrin in these strains, the frequency of a mutated sodium channel gene was determined using a PCR assay. The tick strains from Mexico and Brazil demonstrated 49.4- to over 672.2-fold resistance to permethrin, and up to 94.5-fold resistance to amitraz. While the San Roman strain from Mexico was the most permethrin-resistant strain, the Santa Luiza strain from Brazil was the most amitraz-resistant strain. A significant correlation was found between the permethrin resistance ratio and the allelic frequency of the sodium channel mutation. Significant synergism between permethrin and amitraz was found when one acaricide was tested in the presence of another. Synergism ratios ranged from 1.5 to 54.9 when amitraz was tested as a synergist for permethrin. Similar synergism ratios were obtained when permethrin was tested as a synergist for amitraz. Permethrin caused virtually no mortality in the San Roman strain, even at the highest concentration (3294 μg cm^{-2}). Adding amitraz (11.0 μg cm^{-2}) to permethrin led to a dramatic increase in larval mortality, even at very low concentrations of permethrin.

Keywords: acaricide; synergism; permethrin; amitraz; cattle tick; sodium channel mutation

1 INTRODUCTION

The southern cattle tick, *Boophilus microplus* (Canestrini), is a damaging ectoparasite of cattle and the key vector of bovine babesioses (Texas fever) which once devastated the US cattle industry.[1,2] This pest was eradicated from the southern United States in the 1940s after an intensive eradication campaign that lasted over three decades.[1,3,4] The US Department of Agriculture (USDA) has since maintained an active cattle fever tick eradication program (CFTEP) along the US–Mexican border to prevent the reintroduction of *B. microplus* via cattle exported to the USA from Mexico, where *B. microplus* remains endemic and continues to cause serious economic damage. One critical component of the CFTEP is the systematic treatment of all cattle imported from Mexico in total immersion vats charged with coumaphos, an organophosphate (OP) acaricide, to eliminate ticks that the cattle may carry. In Mexico, *B. microplus* has developed resistance to coumaphos and other acaricides in past decades owing to intensive use of chemical acaricides.[3,5–9] Resistance to OP acaricides first developed in the 1980s in Mexico, and resistance to pyrethroids emerged in the 1990s.[5,10,11] Amitraz, a formamidine acaricide, was introduced along with pyrethroids to control OP-resistant ticks in Mexico in 1986.[10,12] Initially, amitraz was not widely used owing to its higher cost, but its use became more prevalent and intensive after pyrethroid resistance was discovered in 1993.[13] The first case of amitraz resistance in *B. microplus* from Mexico was confirmed in 2001 at a ranch in the state of Tabasco.[12] Presently, many tick populations are resistant to multiple classes of acaricides in Mexico.[14]

Different pesticide resistance management strategies, including rotation of pesticides, and mixtures of

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pesticides or synergists, have been reported to be effective in controlling resistant insect pests.[15–20] However, acaricide rotation may no longer be a good option for tick control in some areas of Mexico, as many tick populations have developed resistance to multiple classes of acaricides, including OP, pyrethroid and amitraz. Alternatively, there has been success in the application of mixtures of insecticides to suppress insect pests that are resistant to the application of a single insecticide. For example, the whitefly, *Bemisia tabac*i Gennadius, a serious pest of cotton, melons and vegetables in Arizona, developed high levels of resistance to both pyrethroid and OP insecticides as a result of heavy use of such compounds.[21] To overcome the resistance problem, mixtures of various insecticides with different modes of action were evaluated to control resistant white flies.[19,22] An increase of over 1000-fold in the toxicity of fenpropathrin, a pyrethroid, to *B. tabac*i was observed when acephate, an OP insecticide, was co-applied with the pyrethroid.[19] The mixture formulation of fenpropathrin and acephate provided an effective control to highly resistant *B. tabaci* populations when it was adopted by cotton growers in Arizona.[22] Similarly, mixtures of OP and pyrethroid insecticides have been shown to be effective in the control of the cotton bollworm, *Helicoverpa armigera* (Hübner), and the southern house mosquito, *Culex quinquefasciatus* Say.[20,23]

Although OPs, pyrethroids and amitraz have now been used widely to control *B. microplus* in Mexico, the potential of the acaricide mixture strategy for tick control has not yet been explored. Synergism between pyrethroid and formamidine insecticides has been previously reported in several insect species.[24,25] It is unknown whether such synergism exists between these two classes of acaricides in ticks. The authors have maintained in the laboratory a susceptible strain and several resistant strains of *B. microplus* that originated from Mexico and Brazil. These tick strains provided an opportunity for detailed studies on resistance mechanisms and possible interaction between different classes of acaricides in *B. microplus*. The objectives of this study were to determine levels of resistance to permethrin and amitraz in *B. microplus*, correlate allelic frequencies of a sodium channel mutation that confers pyrethroid resistance with levels of resistance determined by bioassays and evaluate synergism between permethrin and amitraz to assess its usefulness in tick resistance management.

2 MATERIALS AND METHODS

2.1 Ticks

Five strains of *B. microplus* were used in this study. The Muñoz strain was established at the USDA Cattle Fever Tick Research Laboratory (CFTRL) in 1999 from an outbreak of *B. microplus* ticks in Zapata County, Texas. The Muñoz strain was susceptible to all major classes of acaricides tested, and therefore was used as a susceptible reference strain to determine the level of resistance in other tick strains.[7,26,27] The OP-resistant Pesqueria strain was collected in 2000 at the US port of entry in Reynosa, Tamaulipas, Mexico, by USDA Veterinary Service inspectors from cattle originating in Pesqueria, Nuevo Leon, Mexico. The Santa Luiza strain was an amitraz-resistant tick strain collected from a ranch in Brazil, and was maintained at the Mexican National Parasitology Laboratory, Jiutepec, Morelos, Mexico, before being established at CFTRL in Mission, Texas, in 2000. The OP-resistant San Roman strain was collected from a ranch in Champoton, Campeche, Mexico, and was established at the CFTRL in 1998. The pyrethroid-resistant San Felipe strain was collected from a ranch in the state of Tamaulipas, Mexico, and was established at the CFTRL in 1996. The San Felipe strain was challenged with permethrin, the San Roman strain with coumaphos, the Santa Luiza strain with amitraz and the Pesqueria strain with diazinon to increase or maintain resistance to the respective acaricides during their laboratory colonization and maintenance.[6–8] The procedures for rearing ticks on cattle, maintaining non-parasitic stages in the laboratory and challenging larvae with acaricides were similar to those previously described.[28,29]

2.2 Chemicals

Amitraz 125 g L^{-1} EC (Taktic®; NOR-AM Chemical Company, Wilmington, DE) was used in this study. Technical-grade permethrin [92.2% active ingredient (AI); *cis:trans* ratio = 1:3] was obtained from FMC (Philadelphia, PA). Three synergists used in this study, triphenylphosphate (TPP) (an inhibitor of esterases), piperonyl butoxide (PBO) (an inhibitor of cytochrome P450 monooxygenases) and diethyl maleate (DEM) (an inhibitor of glutathione-*S*-transferases), were purchased from Aldrich (Milwaukee, WI).

2.3 Larval bioassays

A slightly modified version of the larval packet test (LPT) recommended by FAO[30] was used to determine permethrin toxicity to tick larvae, levels of permethrin resistance and the effect of synergists on permethrin toxicity.[6] Larvae that were 12–16 days old were used for all bioassays. A stock solution of permethrin was made by dissolving technical-grade permethrin in trichloroethylene (Sigma, St Louis, MO). The top concentration was prepared by adding a volume of the stock solution to a mixture of trichloroethylene and olive oil (Sigma) with a final 2:1 ratio. Serial dilutions from the top concentration were made using a diluent of two parts trichloroethylene and one part oil. A volume of 0.7 mL of each dilution was applied to a Whatman No. 1 filter paper (7.5 × 8.5 cm; Whatman, Maidstone, Kent, UK). Three filter papers were prepared for each dilution. Treated filter papers were placed in a fume hood for 2 h, to allow trichloroethylene to evaporate, before being folded in half and sealed with bulldog clips on both sides. Approximately 100 larvae were placed into each

packet, and the top was sealed immediately with another bulldog clip. Packets were then held in an environmental chamber at 27 ± 2 °C, 90% RH for 24 h. Packets were removed from the environmental chamber, and mortality was determined by counting live and dead larvae.

A modified FAO larval packet test (LPT)[8,26,31] was used for all amitraz bioassays in this study. Pieces (7.5 × 8.5 cm) of nylon fabric (type 2320; Cerex Advanced Fabrics, Pensacola, FL) were used as the substrate instead of the Whatman filter papers.

2.4 Synergism study

When amitraz was evaluated as a synergist for permethrin, the modified FAO bioassay technique for permethrin was used. A concentration of amitraz that would cause <25% mortality was first determined with the filter paper bioassay technique for each tick strain. The appropriate concentration of amitraz was then used as the diluent to prepare serial dilutions of permethrin for a particular tick strain. When permethrin was evaluated as a synergist for amitraz, the modified FAO bioassay technique with nylon fabric as substrate for amitraz was used. A concentration of permethrin that would cause little or low larval mortality was first determined with the nylon fabric bioassay technique for each tick strain. The permethrin solution was then used as the diluent to prepare serial dilutions of amitraz for a particular tick strain.

Effects of three synergists, TPP, PBO and DEM, on permethrin toxicity to *B. microplus* larvae were evaluated only in the San Roman strain by adding one of the synergists to the diluent to achieve a constant concentration of 109.8 μg cm^{-2} on substrate, the highest concentration at which no larval mortality in *B. microplus* was observed when applied alone (unpublished data). Diluent with that constant concentration of the synergist was used to make serial dilutions of permethrin.

2.5 Detection of the sodium channel mutation

Larvae used to detect the sodium channel mutation were either from the same generation when bioassays were conducted or from generations immediately before or after. Tick larvae were individually ground in 20 μL of 0.1× TE (Tris-EDTA, 0.1–1 mM, pH 8.0) buffer in microcentrifuge tubes with polypropylene pestles for approximately 30 s. A quantity of 100 μL of Chelex (BioRad Laboratories, Hercules, CA; 5% suspension in 0.1× TE buffer) was immediately added, incubated for 15 min at 55 °C, then at room temperature for 30 min and finally centrifuged for 5 min at 12 000 × *g* at room temperature. The supernatant was removed as the DNA template and stored at −20 °C until use. For detection of a T → A mutation conferring pyrethroid resistance in *B. microplus*, a gel-based PCR assay was used.[32,33]

2.6 Data analysis

Probit analysis of dose–mortality data was performed using POLO-PC.[34] The resistance ratio (RR) was calculated by dividing the LC_{50} (μg cm^{-2}) of a particular tick strain by the LC_{50} of the reference Muñoz strain. The synergism ratio (SR) of one acaricide caused by another acaricide was calculated by dividing the LC_{50} of the bioassay using one acaricide alone by the LC_{50} using the mixture of that acaricide and the other acaricide (synergist). The difference between LC_{50} estimates was designated as significant if the 95% confidence intervals (CIs) did not overlap. Mean mortalities at the same acaricide concentrations with and without the presence of the other acaricide or a synergist were compared with the *t*-test using the JMP software.[35]

3 RESULTS

3.1 Resistance to permethrin

Results of probit analysis of concentration–mortality data of permethrin with and without amitraz in the five strains of *B. microplus* are summarized in Table 1. Compared with the susceptible Muñoz strain, all Mexican strains and the Brazilian strain (Santa Luiza) had varying levels of resistance to permethrin, with RRs ranging from 49.4 to >672.2. The San Roman

Table 1. Resistance to permethrin and synergism of permethrin toxicity to tick larvae by amitraz in various strains of *Boophilus microplus*

Tick strain	Acaricides	*n*	Slope (± SE)	χ^2 (df)	LC_{50} (μg cm^{-2}) (CI)[a]	RR[b]	SR[c]
Muñoz	Permethrin	1639	3.2(±0.2)	32.8 (19)	4.9 (4.4–5.5)	1.0	
	+ amitraz (0.4 μg cm^{-2})	1550	4.7(±0.3)	55.4 (16)	2.4 (2.1–2.7)*		2.0
Pesqueria	Permethrin	2467	0.9(±0.1)	61.5 (19)	241.8 (190.3–338.2)	49.4	
	+ amitraz (2.8 μg cm^{-2})	2379	1.0(±0.1)	41.8 (19)	129.9 (89.2–179.7)*		1.9
Santa Luiza	Permethrin	2164	4.6(±0.2)	162.5 (6)	444.5 (382.8–601.1)	90.7	
	+ amitraz (11.0 μg cm^{-2})	1937	8.6(±0.4)	42.0 (15)	178.4 (168.8–188.1)*		2.5
San Felipe	Permethrin	1410	0.7(±0.1)	45.9 (19)	3125.8 (1639.5–8354.9)	637.9	
	+ amitraz (1.1 μg cm^{-2})	1181	0.9(±0.1)	53.8 (19)	2127.3 (1076.3–5150.7)		1.5
San Roman	Permethrin	2435	–	–	>3294.0	>672.2	
	+ amitraz (11.0 μg cm^{-2})	2793	–	–	~60.0*		~54.9

[a] CI = confidence interval.
[b] RR = resistance ratio.
[c] SR = synergism ratio.
* indicates significant difference from permethrin alone.

strain demonstrated the highest permethrin resistance. There was virtually no mortality when larvae were exposed to the highest concentration (3294 $\mu g\,cm^{-2}$) of permethrin used in the tick bioassay.

3.2 Frequency of the sodium channel mutation

Results of PCR analysis of tick larvae in all five strains of *B. microplus* utilized are summarized in Table 2. The susceptible Muñoz strain had few larvae carrying a copy of the mutated gene. The allelic frequency of the sodium channel mutation was the lowest (7.3%) among all strains, and the mutated copy only appeared in a heterozygous form. The San Roman strain had the highest allelic frequency of the mutated gene (100%), and all larvae tested were homozygous for the mutation. The Pesqueria strain had a mutated allelic frequency of 42.7%, and most appeared in the heterozygous form. No copies of the mutated gene were detected in the Santa Luiza strain, although this tick strain demonstrated a 90.7-fold resistance to permethrin. Overall, there was a significant correlation between the LC_{50} estimates and the sodium channel mutation frequencies ($r^2 = 0.827$, $P < 0.05$).

3.3 Effects of amitraz and other synergistic compounds on permethrin toxicity

Amitraz alone, at a given concentration (see Table 1), caused no mortality in the Muñoz strain, very low mortality (2.4%) in the Santa Luiza strain and higher mortalities in other strains (12.6%, 15.3% and 22.1% in Pesqueria, San Felipe and San Roman strains respectively). The addition of amitraz to permethrin produced increased toxicity of permethrin to tick larvae in all strains, with SRs ranging from 1.5 to >54.9. The most significant synergism of permethrin toxicity by amitraz was observed in the San Roman strain (Table 1). Permethrin alone, even at the highest concentration used (3294 $\mu g\,cm^{-2}$), caused virtually no mortality in this strain. None of the traditional synergists tested (TPP, PBO and DEM) had any effect on permethrin toxicity in the San Roman strain (data not shown). The inclusion of amitraz (11 $\mu g\,cm^{-2}$) in the permethrin bioassay dramatically increased permethrin toxicity in the San Roman strain. When PBO (109.8 $\mu g\,cm^{-2}$) was added to diluent containing amitraz (11 $\mu g\,cm^{-2}$) for permethrin bioassay, further increase in tick mortality was observed, particularly at low permethrin concentrations tested (12.9–51.5 $\mu g\,cm^{-2}$) (Table 3).

Table 2. Allelic frequency of the sodium channel mutation (*scm*) detected with the PCR assay in various strains of *Boophilus microplus*

Tick strain	Generation	*n*	Genotype SS	SR	RR	Allelic *scm* frequency (%)
Muñoz	f-19	48	41	7	0	7.3
Pesqueria	f-14	48	10	35	3	42.7
Santa Luiza	f-13	48	48	0	0	0.0
San Felipe	f-41	48	4	11	33	80.2
San Roman	f-30	48	0	0	48	100.0

Table 3. Effects of amitraz and PBO on permethrin toxicity in larvae of the San Roman strain of *Boophilus microplus*

Permethrin concentration ($\mu g\,cm^{-2}$)	Mortality (%) (± SD) Permethrin	Permethrin + amitraz[a]	Permethrin + amitraz[a] + PBO[b]
3294.0	1.2(±1.4)	87.7(±8.6)	75.4(±9.4)
1647.0	1.5(±2.6)	68.0(±17.7)	83.2(±15.1)
823.5	0	73.6(±6.0)	78.1(±1.4)
411.8	1.0(±1.7)	72.4(±23.0)	72.3(±13.3)
205.9	0	68.5(±11.8)	62.7(±15.5)
102.9	1.2(±2.0)	78.7(±5.1)	77.1(±13.0)
51.5	0	44.3(±13.1)	72.9(±7.2)*
25.7	0	6.2(±2.6)	62.1(±4.7)*
12.9	0	4.6(±2.7)	65.5(±7.1)*
0	0	22.1(±7.3)	42.0(±31.5)*

[a] Amitraz concentration = 11.0 $\mu g\,cm^{-2}$.
[b] PBO concentration = 109.8 $\mu g\,cm^{-2}$.
* indicates significant difference from other treatments in row (*t*-test, $P < 0.001$).

3.4 Resistance to amitraz

The results of probit analysis of concentration–mortality data of amitraz with and without permethrin in five strains of *B. microplus* included in this study are summarized in Table 4. Compared with the susceptible reference strain (Muñoz strain), the San Roman and the San Felipe strains were slightly less susceptible to amitraz (RR = 2.0 and 1.4 respectively), the Pesqueria strain was moderately resistant (RR = 18.0) and the Santa Luiza strain was the most resistant (RR = 94.5).

3.5 Effects of permethrin on amitraz toxicity

When permethrin was tested as a synergist of amitraz, permethrin alone (2.2 $\mu g\,cm^{-2}$) caused very high (63%) mortality in the Muñoz strain, and therefore the data were excluded. No bioassay was conducted to test permethrin as a synergist for amitraz in the San Roman strain. In other tick strains evaluated, permethrin alone, at a given concentration (see Table 4), caused very low mortality (2.5–3.1%) in the Pesqueria and Santa Luiza strains, and a slightly higher mortality (21.9%) in the San Felipe strain. The addition of permethrin to amitraz resulted in increased toxicity of amitraz to tick larvae in all strains, with SRs ranging from 1.5 to 54.0. The most dramatic synergism of amitraz toxicity by permethrin was observed in the Santa Luiza strain (Tables 4 and 5). Amitraz alone caused virtually no mortality at concentrations of 6.9 $\mu g\,cm^{-2}$ and lower. When permethrin (109.8 $\mu g\,cm^{-2}$) was added to amitraz bioassay, the same amitraz concentration (6.9 $\mu g\,cm^{-2}$) produced 85.3% mortality.

Table 4. Resistance to amitraz and synergism of amitraz toxicity to tick larvae by permethrin in various strains of *Boophilus microplus*

Tick strain	Acaricides	*n*	Slope (± SE)	χ^2 (df)	LC_{50} (μg cm^{-2}) (CI)[a]	RR[b]	SR[c]
Muñoz	Amitraz	2265	1.6(±0.1)	311.0 (22)	0.4 (0.1–0.7)	1.0	
San Roman	Amitraz	1969	1.9(±0.1)	54.2 (22)	0.8 (0.7–1.0)*	2.0	
San Felipe	Amitraz	1626	2.9(±0.2)	33.6 (13)	0.6 (0.5–0.7)	1.5	
	+ permethrin (549 μg cm^{-2})	1465	1.6(±0.2)	53.0 (13)	0.1 (0.0–0.2)*		6.0
Pesquria	Amitraz	1811	1.9(±0.1)	43.3 (22)	7.2 (6.0–8.4)	18.0	
	+ permethrin (2.8 μg cm^{-2})	2110	2.1(±0.1)	68.0 (22)	4.9 (4.0–5.9)*		1.5
Santa Luiza	Amitraz	2019	2.2(±0.1)	181.6 (19)	37.8 (27.4–50.2)	94.5	
	+ permethrin (109.8 μg cm^{-2})	2660	1.1(±0.1)	162.9 (25)	0.7 (0.3–1.3)*		54.0

[a] CI = confidence interval.
[b] RR = resistance ratio.
[c] SR = synergism ratio.
* indicates significant difference from amitraz alone.

Table 5. Effects of permethrin on amitraz toxicity in larvae of the Santa Luiza strain of *Boophilus microplus*

	Mortality (%) (± SD)	
Amitraz concentration (μg cm^{-2})	Amitraz	Amitraz + permethrin[a]
219.6	100.0(±0.0)	100.0(±0.0)
109.8	88.8(±0.5)	98.5(±1.8)*
54.9	57.7(±23.7)	98.3(±2.9)*
27.5	39.6(±12.3)	96.3(±4.2)*
13.7	28.6(±26.3)	94.0(±3.5)*
6.9	2.6(±1.1)	85.3(±4.5)*
3.4	5.7(±7.5)	60.4(±12.6)*
1.7	3.4(±2.6)	61.8(±27.1)*
0.9	–	70.5(±12.7)*
Permethrin only	–	3.1(±1.6)
0	0.3(±0.5)	1.5(±2.6)

[a] Permethrin concentration = 109.8 μg cm^{-2}.
* indicates significant difference from other treatment in row (*t*-test, $P < 0.001$).

4 DISCUSSION

Results from this and previous studies have shown that resistance to pyrethroids and amitraz coexists in the tick strains from Mexico and Brazil. Double or possibly triple resistance to all three major classes of acaricides (OPs, pyrethroids and amitraz) has become increasingly prevalent in Mexico,[7,8,14] and mechanisms of resistance to these commonly used acaricides have been extensively studied in recent years.[36,37] Both the sodium channel mutation and metabolic detoxification mechanisms are known to be responsible for pyrethroid resistance.[32,36,37] Although a significant correlation was found between the permethrin resistance ratio and the allelic frequency of the sodium channel mutation in this study, metabolic mechanisms of resistance, instead of insensitive target sites, were presumed to play a major role in Santa Luiza strain owing to the lack of a sodium channel mutation. Results of further synergist bioassays with TPP, PBO and DEM suggest the existence of enhanced metabolic detoxification mechanisms involving esterases and mixed-function oxidases (Li *et al.*, unpublished data). The San Felipe strain was described previously by Miller *et al.*[6] as having >1000-fold resistance to permethrin. He *et al.*[32] identified a sodium channel mutation in this and another highly pyrethroid-resistant strain (Corrales). This led to the development of a PCR assay for detecting this resistance gene.[33] Since its laboratory colonization, this tick strain has maintained a high level of pyrethroid resistance and a high allelic frequency of the mutated sodium channel gene (80.2%), similar to that previously reported.[33] Challenges of larvae with permethrin in the laboratory over the years failed to eliminate wild-type susceptible allele from this tick strain. The failure to eliminate the susceptible allele from this tick strain was likely caused by the relatively low concentration (109.8 μg cm^{-2}) of permethrin used, which was apparently sufficient to maintain resistance level but not high enough to eliminate all heterozygotes possessing the susceptible allele.

The San Roman strain has been the most OP-resistant tick strain and has been repeatedly challenged to maintain its high level of resistance to coumaphos since its laboratory colonization. Although this tick strain has not been exposed to pyrethroids since its collection from Mexico in 1998, it has maintained a very high level of resistance to permethrin. The highest level of permethrin resistance in the San Roman strain is well supported by PCR data indicating 100% frequency of homozygous mutant alleles (Table 2). The total lack of synergism of permethrin toxicity by the three traditional synergists (TPP, PBO and DEM) suggests that metabolic detoxification is not involved in permethrin resistance in this particular tick strain, and a sodium channel mutation is the sole mechanism conferring permethrin resistance. It is likely that individual ticks collected from Mexico for the establishment of the San Roman strain were all homozygous resistant genotype (RR) for the sodium channel mutation. The mutated gene was fixed and remained stable in the colony even without pressure of selection with permethrin during the entire period of its laboratory colonization (>30 generations).

Compared with permethrin or amitraz alone, enhanced toxicity by one acaricide was observed when the other acaricide was added in both susceptible and resistant tick strains. To test possible synergistic

effects of mixtures of these two acaricides, only concentrations of permethrin or amitraz that caused relatively low mortalities (<25%) were used when it was tested as a synergist.

It should be pointed out that probit analysis may be problematic when the 'control mortality' was higher than 10% in bioassays where one acaricide was used as a 'synergist' for the other acaricide. Also, because different concentrations of the 'synergist acaricide' were used for different tick strains, it may not be appropriate directly to compare the SRs of the same acaricide among the tick strains. Nevertheless, adding amitraz to permethrin or permethrin to amitraz significantly increased the toxicity of permethrin or amitraz alone to tick larvae in both susceptible and resistant strains. Results from this study provide positive confirmation of the synergism between permethrin and amitraz in larvae of *B. microplus*.

Although the mode of action of pyrethroids is general knowledge, the mode of action of amitraz is not well defined. It has been proposed that amitraz and other formamidine pesticides exert their toxic effect on pest species by binding to the octopamine receptor of the central nervous system, and possibly also by inhibition of monoamine oxidases.[38,39] Synergistic effects of formamidines on pyrethroids have been previously reported in the tobacco budworm, *Heliothis virescens* F., and the housefly, *Musca domestica* L.[40–42] Three different mechanisms by which synergism between formamidine and pyrethroid insecticides occurs have been proposed for these insect species.[42–45] Synergism of pyrethroids by chlordimeform, a formamidine, in the tobacco budworm was thought to be caused by enhanced uptake of pyrethroids as a result of exposure to chlordimeform.[43,44] The same mechanism was also suggested for the housefly, where formamidine exposure of houseflies caused a 2.5-fold increase in uptake of a radiolabeled pyrethroid.[42] Liu and Plapp[45] conducted a further study on houseflies with and without the *kdr* mutation, by measurements of radiolabeled Saxitoxin (STX) binding to the nerve membrane sodium channel from untreated and chlordimeform-treated flies. They found that formamidines reduced the number of STX binding components and decreased the concentration at which saturation of STX binding occurred. They concluded that formamidines act as target-site synergists of pyrethroids by modifying binding cooperativity in target tissues. The third mechanism was proposed by Usmani *et al*.[24] who reported that amitraz pretreatment of the bollworm, *Helicoverpa zea* (Boddie), before treatment of third-instar larvae with permethrin resulted in a decreased rate of pyrethroid metabolism when compared with larvae treated with permethrin alone. *In vitro* metabolism experiments also confirmed that amitraz and some of its metabolites inhibited degradation of permethrin.[25]

In the present study, the authors were unable to determine the exact mechanisms by which synergism between permethrin and amitraz occurred. However, it was demonstrated that amitraz ($11.0\,\mu g\,cm^{-2}$) dramatically synergized permethrin toxicity in the San Roman strain, the most permethrin-resistant strain with 100% homozygous resistant genotype. Larval mortality quickly reached a plateau (around 70–80%), but never reached 100% even at the highest permethrin concentration tested (Table 3). A similar pattern was also observed when permethrin was tested as a synergist for amitraz (Table 5). When PBO was added to the permethrin and amitraz mixture, even higher tick mortalities were observed at low permethrin concentrations ($12.9–51.5\,\mu g\,cm^{-2}$) (Table 3). PBO has been shown to synergize amitraz toxicity to tick larvae,[8] possibly by inhibiting oxidase-based detoxification of amitraz. Amitraz itself has been shown to inhibit monoamine oxidase in *B. microplus*,[39] which was regarded as one possible mode of action of amitraz. It is reasonable to suggest that the synergism of permethrin and amitraz mixture was further increased by PBO through PBO inhibition of oxidase activity in tick larvae, leading to reduced metabolism of amitraz and/or permethrin.

Synergism between permethrin and amitraz reported in this study may be caused by one or more of the three mechanisms proposed by previous researchers. Regardless, the finding of synergism between permethrin and amitraz in tick larvae has significant implications for the management of acaricide resistance in *B. microplus*. New formulations of permethrin and amitraz mixture could be used to control tick populations that are highly resistant to one or both acaricides. However, the ratio of each component in the mixture has to be further tested and determined on the basis of field efficacy trials. The acaricide mixture strategy should be approached with caution as it has been demonstrated in some insects that targeted pests can also develop resistance to insecticide mixtures.[22]

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REFERENCES

1 Graham OH and Hourrigan JL, Eradication program for the arthropod parasites of livestock. *J Med Entomol* **6**:629–658 (1977).

2 Friedhoff KT and Smith RD, Transmission of *Babesia* by ticks, in *Babesiosis*, ed. by Ristic M and Kreier JP. Academic Press, New York, pp. 267–321 (1981).

3 George JE, The campaign to keep *Boophilus* ticks out of the United States: technical problems and solutions. *Proc 100th Annual Meeting of the US Animal Health Association*, Spectrum, Richmond, VA, pp. 196–206 (1996).

4 Bram RA, George JE, Reichard RE and Tabachnick WJ, The threat of foreign arthropod-borne pathogens to livestock in the United States. *J Med Entomol* **39**:405–416 (2002).

5 Santamaría VM, Soberanes CN, Ortiz NA, Fragoso SH, Osorio MJ, Martínez IF, *et al*, Analisis de la situacion actual ediante el monitoreo de susceptibilidad a ixodicidas en *Boophilus microplus* de 1993 a 1999 y medidas preventivas para retardar la resistencia al amitraz en Mexico. *IV Seminario Internacional de Parasitologia Animal: Control de la Resistancia en Garrapatas y Moscas de Importancia Veterinaria y Enfermedades que Transmiten*, Puerto Vallarta, Jalisco, Mexico, 20–22 October 1999, pp. 103–117 (1999).

6 Miller RJ, Davey RB and George JE, Characterization of pyrethroid resistance and susceptibility to coumaphos in Mexican *Boophilus microplus* (Acari: Ixodidae). *J Med Entomol* **36**:633–538 (1999).

7 Li AY, Davey RB, Miller RJ and George JE, Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism. *J Med Entomol* **40**:482–490 (2003).

8 Li AY, Davey RB, Miller RJ and George JE, Detection and characterization of amitraz resistance in the southern cattle tick *Boophilus microplus* (Acari: Ixodidae). *J Med Entomol* **41**:482–490 (2004).

9 Kunz SE and Kemp DH, Insecticides and acaricides: resistance and environmental impacts. *Rev Sci Tech Off Ini Epiz* **13**:1249–1286 (1994).

10 Aguirre J, Sobrino A, Santamaría M, Aburto A, Roman S, Hernandez M, *et al*, Resistancia de garrapatas en Mexico. *Seminario Internacional de Parasitologia Animal*, Cuernavaca, Morelos, Mexico, 8–10 September 1986, ed by Caravazzai AH and Garcia Z. pp. 282–306 (1986).

11 Fragoso H, Soberanes N, Ortiz M, Santamaría M and Ortiz A, Epidemiologia de la resistencia a ixodicidas piretroides en garrapatas *Boophilus microplus* en la Republica Mexicana. *Seminario Internacional de Parasitologia Animal – Resistancia y Control en Garrapatas y Moscas de Importancia Veterinaria*, Acapulco, Guerrero, Mexico, ed. by Rodriguez S and Fragoso H. pp. 45–57 (1995).

12 Soberanes NC, Santamaría MV, Fragoso HS and García ZV, First case reported of amitraz resistance in the cattle tick *Boophilus microplus* in Mexico. *Téc Pecu Méx* **40**:81–92 (2002).

13 Parrodi LF, Fragoso HY and Nunez JL, Amitraz: Una alternativa para el control de garrapatas *Boophilus* spp. resistentes a organofosforados y pirethroides. *Seminario Internacional de Parasitologia Animal – Resistancia y Control en Garrapatas y Moscas de Importancia Veterinaria*, Acapulco, Guerrero, Mexico, ed. by Rodriguez S and Fragoso H. p. 146 (1995).

14 Rodriguez-Vivas I, Prevalence and potential risk factors for amitraz resistance in *Boophilus microplus* ticks in cattle from the state of Yucatan, Mexico. *V International Seminar in Animal Parasitology: World Situation of Parasite Resistance in Veterinary Medicine,* SENASICA-INIFAP-INFARVET-USDY-FAO-AMPAVE, Merida, Yucatan, México, 1–3 October 2003, ed. by García ZV and Fragoso HS. pp. 32–36 (2003).

15 Curtis CF, Theoretical models of the use of insecticide mixtures for the management of resistance. *Bull Entomol Res* **75**:259–265 (1985).

16 Byford RL, Lockwood JA, Smith SM, Sparks TC and Luther DG, Insecticide mixtures as an approach to the management of pyrethroid-resistant horn flies (Diptera: Muscidae). *J Econ Entomol* **80**:111–116 (1987).

17 Sparks TC and Byford RL, Pyrethroid–synergist mixtures: toxicity, resistance, and field efficacy toward pyrethroid-resistant horn flies (Diptera: Muscidae). *J Econ Entomol* **81**:1567–1574 (1988).

18 Mckenzie CL and Byford RL, Continuous, alternating, and mixed insecticides affect development of resistance in the horn fly (Diptera: Muscidae). *J Econ Entomol* **86**:1040–1048 (1993).

19 Wolfebarger DA, Riley DG, Akey DH and Jones WA, Endosulfan and bifenthrin in alternate applications and mixture against B-strain whitefly. *Proceedings of the Beltwide Cotton Conference*, National Cotton Council of America, Memphis, TN, pp. 901–903 (1994).

20 Corbel V, Raymond M, Chandre F, Darriet F and Hougard J-M, Efficacy of insecticide mixtures against larvae of *Culex quinquefasciatus* (Say) (Diptera: Culicidae) resistant to pyrethroids and carbamates. *Pest Manag Sci* **60**:375–380 (2003).

21 Dennehy TJ, Williams L, Russel JS, Li XH and Wigert M, Monitoring and management of whitefly resistance to insecticides in Arizona. *Proceedings of the Beltwide Cotton Conference*, National Cotton Council of America, Memphis, TN, pp. 135–140 (1996).

22 Dennehy TJ and Williams L, Management of resistance in *Bemisia* in Arizona cotton. *Pestic Sci* **51**:398–406 (1997).

23 Martin T, Ochou OG, Vaissayre M and Fournier D, Organophosphorus insecticides synergize pyrethroids in the resistant strain of cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) from West Africa. *J Econ Entomol* **96**:468–474 (2003).

24 Usmani KA, Abd-Elghafar SF and Knowles CO, Amitraz effect on the pharmacokinetics of permethrin in *Helicoverpa zea* (Lepidoptera: Noctuidae). *J Econ Entomol* **88**:1580–1585 (1995).

25 Liu M and Plapp FW, Mechanism of formamidine synergism of pyrethroids. *Pestic Biochem Physiol* **43**:134–140 (1992).

26 Li AY, Davey RB, Miller RJ and George JE, Mode of inheritance of amitraz resistance in a Brazilian strain of the southern cattle tick *Boophilus microplus* (Acari: Ixodidae). *Exp Appl Acarol* **37**:183–198 (2005).

27 Davey RB, George JE and Miller RJ, Comparison of the reproductive biology between acaricide-resistant and acaricide-susceptible *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Vet Parasitol* **139**:211–220 (2006).

28 Davey RB and George JE, *In vitro* and *in vivo* evaluation of a strain of *Boophilus microplus* (Acari: Ixodidae) selected for resistance to permethrin. *J Med Entomol* **35**:1013–1019 (1998).

29 Davey RB, Garza J Jr, Thompson GD and Drummond RO, Ovipositional biology of the cattle tick, *Boophilus annulatus* (Acari: Ixodidae), in the laboratory. *J Med Entomol* **17**:287–289 (1980).

30 Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides – tentative method for larvae of cattle ticks, *Boophilus microplus* spp. FAO method no. 7. *FAO Plant Protection Bulletin* **19**:15–18 (1971).

31 Miller RJ, Davey RB and George JE, Modification of the food and agriculture organization larval packet test to measure amitraz-susceptibility against Ixodidae. *J Med Entomol* **39**:645–651 (2002).

32 He H, Chen AC, Davey RB, Ivie GW and George JE, Identification of a point mutation in the *para*-type sodium channel gene from a pyrethroid-resistant cattle tick. *Biochem Biophys Res Comm* **261**:558–561 (1999).

33 Guerrero FD, Davey RB and Miller RJ, Use of an allele-specific polymerase chain reaction assay to genotype pyrethroid resistant strains of *Boophilus microplus* (Acari: Ixodidae). *J Med Entomol* **38**:44–50 (2001).

34 *A User's Guide to Probit or Logit Analysis*. LeOra Software, Berkeley, CA (1987).

35 *JMP® Statistics and Graphic Guide, JMP version 4*. SAS Institute, Cary, NC (2000).

36 Guerrero FD, Pruett JH and Li AY, Molecular and biochemical diagnosis of esterase-mediated pyrethroid resistance in a Mexican strain of *Boophilus microplus* (Acari: Ixodidae). *Exp Appl Acarol* **28**:257–264 (2002).

37 Li AY, Use of synergists for elucidation of resistance mechanisms and management of resistant ticks and horn flies. *Proceedings of 30 Años al Servicio de la Ganadería Nacional, 1975–2005*, Jiutepec, Morelos, Mexico, 8–9 June 2005, CD-ROM (2005).

38 Evans PD and Gee JD, Action of formamidine pesticides on octopamine receptor. *Nature (London)* **28**:60–62 (1980).
39 Atkinson PW, Binnington KC and Roulston WJ, High monoamine oxidase activity in the tick *Boophilus microplus* and inhibition by chlordimeform and related pesticides. *J Aus Entomol Soc* **13**:207–210 (1974).
40 Chang CP and Plapp FW, DDT and synthetic pyrethroids mode of action, selectivity, and mechanism of synergism in the tobacco budworm (Lepidoptera: Noctuidae) and a predator, *Chrysopa carnea* Stephens (Neuroptera: Chrysopidae). *J Econ Entomol* **76**:1206–1210 (1983).
41 Campanhola C and Plapp FW, Toxicity and synergism of insecticides against susceptible and pyrethroid-resistant neonate larvae and adults of tobacco budworm (Lepidoptera: Noctuidae). *J Econ Entomol* **82**:1527–1533 (1989).
42 Liu MY and Plapp FW, Formamidines as synergists of cypermethrin in susceptible and pyrethroid resistant house flies (Diptera: Muscidae). *J Econ Entomol* **83**:2181–2186 (1990).
43 Treacy MF, Benedict JH, Schmidt KM, Anderson RM and Wagner TL, Behavior and special distribution patterns of tobacco budworm (Lepidoptera: Noctuidae) larvae on chlordimeform-treated cotton plants. *J Econ Entomol* **80**:1149–1151 (1987).
44 Sparks TC, Leonard BR and Graves JB, Formamidine metabolism and pyrethroid interactions in pyrethroid resistant tobacco budworms. *Proceedings of the Beltwide Cotton Conference*, National Cotton Council of America, Memphis, TN, pp. 330–333 (1989).
45 Liu M-Y and Plapp FW, Mechanism of formamidine synergism of pyrethroids. *Pestic Biochem Physiol* **43**:134–140 (1992).